Review

Cell PRESS

ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering

Thomas Gaj[1,2,3], Charles A. Gersbach[4,5], and Carlos F. Barbas III[1,2,3]

[1] The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA, USA
[2] Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA, USA
[3] Department of Chemistry, The Scripps Research Institute, La Jolla, CA, USA
[4] Department of Biomedical Engineering, Duke University, Durham, NC, USA
[5] Institute for Genome Sciences and Policy, Duke University, Durham, NC, USA

Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) comprise a powerful class of tools that are redefining the boundaries of biological research. These chimeric nucleases are composed of programmable, sequence-specific DNA-binding modules linked to a nonspecific DNA cleavage domain. ZFNs and TALENs enable a broad range of genetic modifications by inducing DNA double-strand breaks that stimulate error-prone nonhomologous end joining or homology-directed repair at specific genomic locations. Here, we review achievements made possible by site-specific nuclease technologies and discuss applications of these reagents for genetic analysis and manipulation. In addition, we highlight the therapeutic potential of ZFNs and TALENs and discuss future prospects for the field, including the emergence of clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases.

Classical and contemporary approaches for establishing gene function

With the development of new and affordable methods for whole-genome sequencing, and the design and implementation of large genome annotation projects, scientists are poised to deliver upon the promises of the genomic revolution to transform basic science and personalized medicine. The resulting wealth of information presents researchers with a new primary challenge of converting this enormous amount of data into functionally and clinically relevant knowledge. Central to this problem is the need for efficient and reliable methods that enable investigators to determine how genotype influences phenotype. Targeted gene inactivation via homologous recombination is a powerful method capable of providing conclusive information for evaluating gene function [1]. However, the use of this technique has been hampered by several factors, including the low efficiency at which engineered constructs are correctly inserted into the chromosomal target site, the need for time-consuming and labor-insensitive selection/screening strategies, and the potential for adverse mutagenic effects. Targeted gene knockdown by RNAi (see Glossary) has provided researchers with a rapid, inexpensive, and high-throughput alternative to homologous recombination [2]. However, knockdown by RNAi is incomplete, varies between experiments and laboratories, has unpredictable off-target effects, and provides only temporary inhibition of gene function. These restrictions impede researchers' ability to link directly phenotype to genotype and limit the practical application of RNAi technology.

Glossary

CRISPR/Cas (CRISPR associated) systems: clustered regulatory interspaced short palindromic repeats are loci that contain multiple short direct repeats, and provide acquired immunity to bacteria and archaea. CRISPR systems rely on crRNA and tracrRNA for sequence-specific silencing of invading foreign DNA. Three types of CRISPR/Cas systems exist: in type II systems, Cas9 serves as an RNA-guided DNA endonuclease that cleaves DNA upon crRNA–tracrRNA target recognition.
crRNA: CRISPR RNA base pairs with tracrRNA to form a two-RNA structure that guides the Cas9 endonuclease to complementary DNA sites for cleavage.
DSB: the product of ZFN, TALEN, and CRISPR/Cas9 action, double-strand breaks are a form of DNA damage that occurs when both DNA strands are cleaved.
HDR: homology-directed repair is a template-dependent pathway for DSB repair. By supplying a homology-containing donor template along with a site-specific nuclease, HDR faithfully inserts the donor molecule at the targeted locus. This approach enables the insertion of single or multiple transgenes, as well as single nucleotide substitutions.
NHEJ: nonhomologous end joining is a DSB repair pathway that ligates or joins two broken ends together. NHEJ does not use a homologous template for repair and thus typically leads to the introduction of small insertions and deletions at the site of the break, often inducing frame-shifts that knockout gene function.
PAM: protospacer adjacent motifs are short nucleotide motifs that occur on crRNA and are specifically recognized and required by Cas9 for DNA cleavage.
RNAi: RNAi is the process by which RNA molecules inhibit or knockdown gene expression. More broadly, RNAi is a natural mechanism that occurs in response to the introduction of many types of RNA molecules into cells.
TALENs: transcription activator-like effector nucleases are fusions of the *Fok*I cleavage domain and DNA-binding domains derived from TALE proteins. TALEs contain multiple 33–35-amino-acid repeat domains that each recognizes a single base pair. Like ZFNs, TALENs induce targeted DSBs that activate DNA damage response pathways and enable custom alterations.
tracrRNA: trans-activating chimeric RNA is noncoding RNA that promotes crRNA processing and is required for activating RNA-guided cleavage by Cas9.
ZFNs: zinc-finger nucleases are fusions of the nonspecific DNA cleavage domain from the *Fok*I restriction endonuclease with zinc-finger proteins. ZFN dimers induce targeted DNA DSBs that stimulate DNA damage response pathways. The binding specificity of the designed zinc-finger domain directs the ZFN to a specific genomic site.
ZFNickases: zinc-finger nickases are ZFNs that contain inactivating mutations in one of the two *Fok*I cleavage domains. ZFNickases make only single-strand DNA breaks and induce HDR without activating the mutagenic NHEJ pathway.

Corresponding author: Barbas, C.F. III (carlos@scripps.edu).
Keywords: zinc-finger nuclease; transcription activator-like effector nuclease; clustered regulatory interspaced short palindromic repeat; genome engineering..

0167-7799/$ – see front matter
 http://dx.doi.org/10.1016/j.tibtech.2013.04.004

In the past decade, a new approach has emerged that enables investigators to manipulate virtually any gene in a diverse range of cell types and organisms. This core technology – commonly referred to as 'genome editing' – is based on the use of engineered nucleases composed of sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module [3,4]. These chimeric nucleases enable efficient and precise genetic modifications by inducing targeted DNA double-strand breaks (DSBs) that stimulate the cellular DNA repair mechanisms, including error-prone nonhomologous end joining (NHEJ) and homology-directed repair (HDR) [5]. The versatility of this approach is facilitated by the programmability of the DNA-binding domains that are derived from zinc-finger and transcription activator-like effector (TALE) proteins. This combination of simplicity and flexibility has catapulted zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) to the forefront of genetic engineering. Here, we review recent advances in site-specific nuclease technologies and discuss applications of these reagents for targeted genome engineering and analysis in eukaryotic cells and model organisms. We also discuss the therapeutic potential of these technologies and examine future prospects, including the development and application of clustered regularly interspaced short palindromic repeats CRISPR/Cas (CRISPR-associated)-based RNA-guided DNA endonucleases.

Custom DNA-binding domains

The versatility of ZFNs and TALENs arises from the ability to customize the DNA-binding domain to recognize virtually any sequence. These DNA-binding modules can be combined with numerous effector domains to affect genomic structure and function (Box 1), including nucleases, transcriptional activators and repressors, recombinases, transposases, DNA and histone methyltransferases, and histone acetyltransferases. Thus, the ability to execute genetic alterations depends largely on the DNA-binding specificity and affinity of designed zinc-finger and TALE proteins. Below, we highlight several of the most successful approaches for assembling these modular DNA-binding domains.

Cys_2–His_2 zinc-finger proteins

The Cys_2–His_2 zinc-finger domain is among the most common types of DNA-binding motifs found in eukaryotes and represents the second most frequently encoded protein domain in the human genome. An individual zinc-finger consists of approximately 30 amino acids in a conserved $\beta\beta\alpha$ configuration [6] (Figure 1A). Several amino acids on the surface of the α-helix typically contact 3 bp in the major groove of DNA, with varying levels of selectivity. The modular structure of zinc-finger proteins has made them an attractive framework for the design of custom DNA-binding proteins. Key to the application of zinc-finger proteins for specific DNA recognition was the development of unnatural arrays that contain more than three zinc-finger domains. This advance was facilitated by the structure-based discovery of a highly conserved linker sequence that enabled construction of synthetic zinc-finger proteins that recognized DNA sequences 9–18 bp in length [7]. Because 18 bp of DNA sequence can confer specificity within 68 billion bp of DNA, this method allowed for specific sequences to be targeted in the human genome for the first time [8,9]. Although initially controversial [10], this design has proven to be the optimal strategy for constructing zinc-finger proteins that recognize contiguous DNA sequences that are specific in complex genomes [6–9,11–15].

Following this proof-of-principle work, several methods for constructing zinc-finger proteins with unique DNA-binding specificity were developed. The 'modular assembly' approach involves the use of a preselected library of zinc-finger modules generated by selection of large combinatorial libraries or by rational design [6,16]. Because zinc-finger domains have been developed that recognize nearly all of the 64 possible nucleotide triplets, preselected zinc-finger modules can be linked together in tandem to target DNA sequences that contain a series of these DNA triplets [6,8,13–15,9]. Alternatively, selection-based approaches, such as oligomerized pool engineering (OPEN) can be used to select for new zinc-finger arrays from randomized libraries that take into consideration context-dependent interactions between neighboring fingers [17]. Approaches have also been developed that combine the methods described above, utilizing zinc-finger modules preselected for con-

Box 1. Beyond nucleases: recombinases, transposases, and transcription factors

Site-specific nucleases are currently the most well-characterized, widely used and broadly applicable tool for inducing custom modifications in cells and model organisms. However, several limitations of targeted nucleases are driving the development of alternative types of programmable enzymes for genome engineering. For example, off-target effects created by site-specific nucleases can be toxic to cells, and difficult to predict and monitor comprehensively. Additionally, because targeted nucleases rely on NHEJ and HDR to induce genetic alterations, this technology may be limited by the availability of the desired DNA repair mechanism in particular cell types. To address these concerns, zinc-finger proteins and TALEs have been fused to enzymatic domains, including site-specific recombinases [28,109–111] and transposases [116], that catalyze DNA integration, excision, and inversion. Because these enzymes perform DNA cleavage and religation autonomously, potentially toxic DNA DSBs should not accumulate in the genome. Additionally, for applications that require targeted gene addition, recombinase and transposase activity is marked by the insertion of donor DNA into the genome, thereby enabling off-target effects to be monitored directly. Moreover, the mechanism of recombination and transposition is independent of cellular DNA repair pathways. As a result, these approaches should be functional in nearly any cell type and cell cycle stage. The efficiency of these processes can also be improved by directed evolution [117]. However, in order for recombinases and transposases to achieve the level of general utility afforded by site-specific nucleases, significant improvements in their performance and flexibility are needed. In particular, recombinase catalytic domains retain sequence specificity from the parental enzyme, and require significant re-engineering toward user-defined DNA targets [109,111]. Although transposase fusions demonstrate high activity at their intended genomic targets, these chimeric proteins also suffer from significant off-target activity [118]. Finally, synthetic zinc-finger and TALE transcription factors offer an alternative approach for inducing targeted modifications by providing stringent control over gene expression [6,8,9,26,27,114,115]. Collectively, these proteins and enzymes represent an exciting suite of tools that can be customized for diverse genome engineering applications.

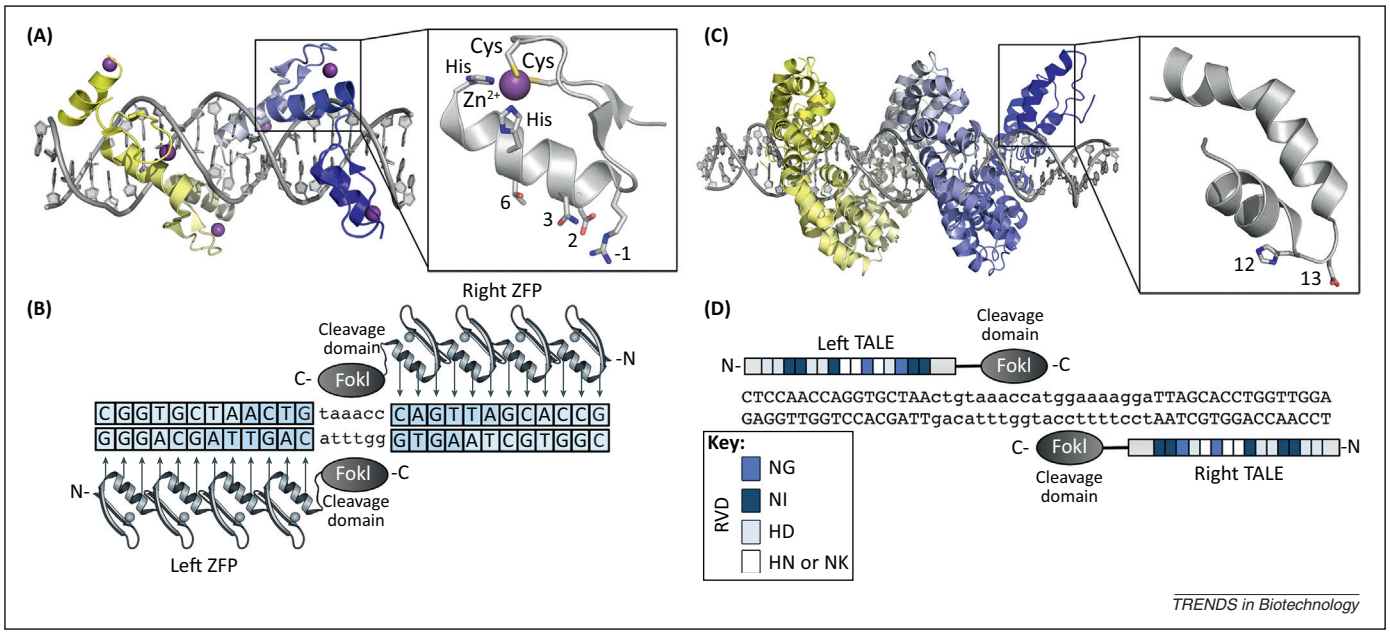

Figure 1. Structure of zinc-finger and transcription activator-like effectors (TALEs). **(A) (Top)** Designed zinc-finger protein in complex with target DNA (grey) (PDB ID: 2I13). Each zinc-finger consists of approximately 30 amino acids in an ββα arrangement (inset). Surface residues (-1, 2, 3 and 6) that contact DNA are shown as sticks. Each zinc-finger domain contacts 3 or 4 bp in the major groove of DNA. The side chains of the conserved Cys and His residues are depicted as sticks in complex with a Zn^{2+} ion (purple). **(B)** Cartoon of a zinc-finger nuclease (ZFN) dimer bound to DNA. ZFN target sites consist of two zinc-finger binding sites separated by a 5–7-bp spacer sequence recognized by the *FokI* cleavage domain. Zinc-finger proteins can be designed to recognize unique 'left' and 'right' half-sites. **(C) (Top)** TALE protein in complex with target DNA (grey) (PDB ID: 3UGM). Individual TALE repeats contain 33–35 amino acids that recognize a single base pair via two hypervariable residues (repeat-variable diresidues; RVDs) (shown as sticks) (inset). **(D)** Cartoon of a TALE nuclease (TALEN) dimer bound to DNA. TALEN target sites consist of two TALE binding sites separated by a spacer sequence of varying length (12–20 bp). TALEs can be designed to recognize unique left and right half-sites. RVD compositions are indicated.

text-dependency to assemble longer arrays by modular assembly [18,19]. For many years, zinc-finger protein technology was the only approach available to create custom site-specific DNA-binding proteins and enzymes. Engineered zinc fingers are also available commercially; Sangamo Biosciences (Richmond, CA, USA) has developed a propriety platform (CompoZr) for zinc-finger construction in partnership with Sigma–Aldrich (St. Louis, MO, USA), allowing investigators to bypass zinc-finger construction and validation altogether, and many thousands of proteins are already available. Broadly, zinc-finger protein technology enables targeting of virtually any sequence.

TALEs

The recent discovery of a simple modular DNA recognition code by TALE proteins [20,21] has led to the explosive expansion of an alternative platform for engineering programmable DNA-binding proteins. TALEs are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains that each recognizes a single base pair (Figure 1B). TALE specificity is determined by two hypervariable amino acids that are known as the repeat-variable di-residues (RVDs) [22,23]. Like zinc fingers, modular TALE repeats are linked together to recognize contiguous DNA sequences. However, in contrast to zinc-finger proteins, there was no re-engineering of the linkage between repeats necessary to construct long arrays of TALEs with the ability of targeting single sites in a genome. Following nearly two decades of pioneering work based on zinc-finger proteins, numerous effector domains have been made available to fuse to TALE repeats for targeted genetic modifications, including nucleases [24–26], transcriptional activators [26,27], and site-specific recombinases [28]. Although the single base recognition of TALE–DNA binding repeats affords greater design flexibility than triplet-confined zinc-finger proteins, the cloning of repeat TALE arrays presents an elevated technical challenge due to extensive identical repeat sequences. To overcome this issue, several methods have been developed that enable rapid assembly of custom TALE arrays. These strategies include 'Golden Gate' molecular cloning [29], high-throughput solid-phase assembly [30,31], and ligation-independent cloning techniques [32]. Several large, systematic studies utilizing various assembly methods have indicated that TALE repeats can be combined to recognize virtually any user-defined sequence [30,32]. The only targeting limitation for TALE arrays for which there is consensus in the literature is that TALE binding sites should start with a T base. Indeed, the ease with which TALE repeats can be assembled is evident in a recent study reporting the construction of a library of TALENs targeting 18 740 human protein-coding genes [33]; a technological feat that will not only facilitate numerous new studies, but will also encourage other, equally ambitious endeavors. Custom-designed TALE arrays are also commercially available through Cellectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island, NY, USA).

Genome editing with site-specific nucleases

Historically, targeted gene inactivation, replacement, or addition has been achieved by homologous recombination; however, the low efficiency of homologous recombination in mammalian cells and model organisms dramatically limits

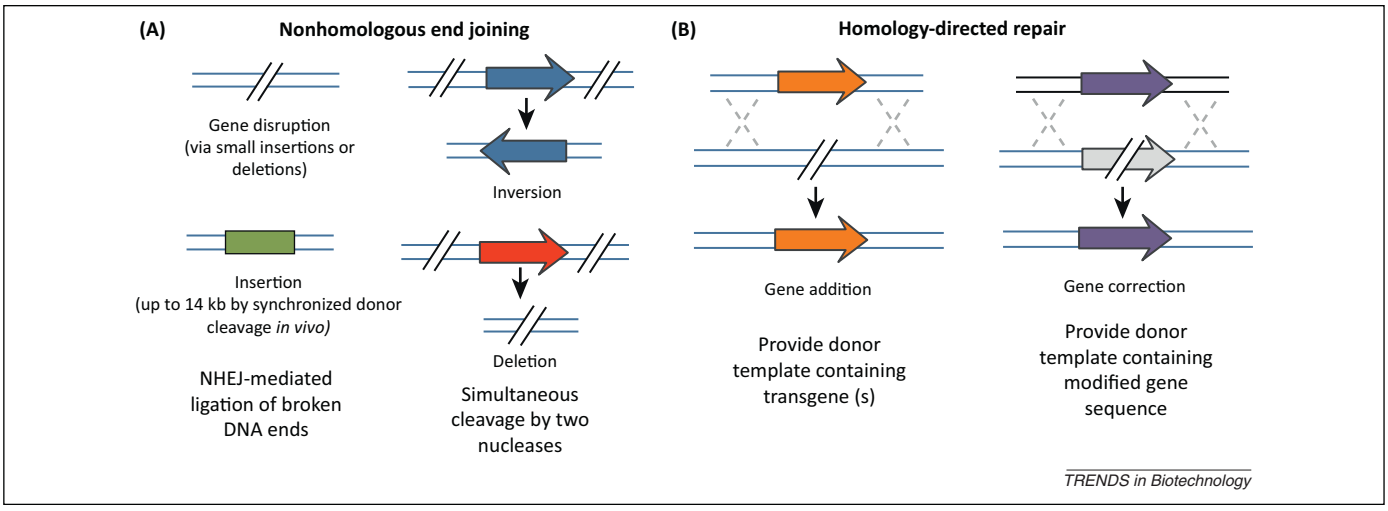

Figure 2. Overview of possible genome editing outcomes using site-specific nucleases. Nuclease-induced DNA double-strand breaks (DSBs) can be repaired by homology-directed repair (HDR) or error-prone nonhomologous end joining (NHEJ). **(A)** In the presence of donor plasmid with extended homology arms, HDR can lead to the introduction of single or multiple transgenes to correct or replace existing genes. **(B)** In the absence of donor plasmid, NHEJ-mediated repair yields small insertion or deletion mutations at the target that cause gene disruption. In the presence of double-stranded oligonucleotides or *in vivo* linearized donor plasmid, DNA fragments up to 14 kb have been inserted via NHEJ-mediated ligation. Simultaneous induction of two DSBs can lead to deletions, inversions and translocations of the intervening segment.

the utility of this approach. Following the discovery that induction of a DSB increases the frequency of HDR by several orders of magnitude, targeted nucleases have emerged as the method of choice for improving the efficiency of HDR-mediated genetic alterations. By co-delivering a site-specific nuclease with a donor plasmid bearing locus-specific homology arms [34], single or multiple transgenes can be efficiently integrated into an endogenous locus (Figure 2A). Linear donor sequences with <50 bp of homology [35], as well as single-stranded DNA oligonucleotides [36], can also be used to induce mutations, deletions, or insertions at the target site. Significantly, nuclease-mediated targeted integration normalizes for positional effects that typically confound many types of genetic analysis and enables study of structure–function relations in the complex and native chromosomal environment. In addition to their role in facilitating HDR, site-specific nucleases also allow rapid generation of cell lines and organisms with null phenotypes; NHEJ-mediated repair of a nuclease-induced DSB leads to the introduction of small insertions or deletions at the targeted site, resulting in knockout of gene function via frameshift mutations [37] (Figure 2B). Site-specific nucleases can also induce deletions of large chromosomal segments [38,39]. This method has been shown to support large chromosomal inversions [40] and translocations [41]. Finally, by synchronizing nuclease-mediated cleavage of donor DNA with the chromosomal target, large transgenes (up to 14 kb) have been introduced into various endogenous loci via NHEJ-mediated ligation [42,43]. Together, these approaches support the study of gene function and the modeling of disease states by altering genes to mimic both known and as yet uncharacterized genotypes. Many of these approaches have been extended to progenitor cell types, including embryonic stem (ES) cells [44] and induced pluripotent stem (iPS) cells [45,46], encouraging their further development for modeling a broad range of genetic conditions [47,48] (Table 1). Extension of this technology to study the role of noncoding DNA in the regulation and expression of coding genes can also be envisioned [49,50], including the use of multiplexed approaches as a means to identify unknown regulatory sites for genes of interest [51].

Improving the performance of site-specific nucleases

In order for customizable nucleases to carry relevance for genetic analysis and clinical application, they must demonstrate strict specificity toward their intended DNA targets. Complex genomes, however, often contain multiple copies of sequences that are identical or highly homologous to the intended DNA target, leading to off-target activity and cellular toxicity. To address this problem, structure [52,53] and selection-based [54,55] approaches have been used to generate improved ZFN and TALEN heterodimers with optimized cleavage specificity and reduced toxicity. Our laboratory has utilized directed evolution to generate a hyperactivated variant of the *Fok*I cleavage domain, *Sharkey*, that exhibits a >15-fold increase in cleavage activity in comparison to traditional ZFNs [55], and is directly compatible with various ZFN architectures [54]. Furthermore, there is mounting evidence to suggest that 4–6 zinc-finger domains for each ZFN half enzyme significantly enhances activity and specificity [13,55–57]. Additional methods for improving ZFN activity include the use of transient hypothermic culture conditions to increase nuclease expression levels [58], co-delivery of site-specific nucleases with DNA end-processing enzymes [59], and the use of fluorescent surrogate reporter vectors that allow for the enrichment of ZFN- and TALEN-modified cells [60]. The specificity of ZFN-mediated genome editing has been further refined by the development of zinc-finger nickases (ZFNickases) [61–63], which take advantage of the finding that induction of nicked DNA stimulates HDR [64] without activating the error-prone NHEJ repair pathway. Consequently, this approach leads to fewer off-target mutagenesis events than conventional DSB-induced methods for genome editing; however, the frequency of HDR by ZFNickases remains lower than those achieved with traditional ZFNs. Finally, conventional DNA- and

Table 1. Abbreviated list of examples of ZFN, TALEN, and CRISPR/Cas-mediated genome editing in human cells and model organisms

Type of modification	Organism	Genes	Nucleases	Refs
Gene disruption	Human	*CCR5*	ZFN	[65,91,92]
			TALEN	[25,52]
			CRISPR/Cas	[101]
	Human	TCR (T cell receptor)	ZFN	[94,95]
	Zebrafish	*gol* (Golden), *ntl* (No tail), *kra*	ZFN	[66,68]
	Pig	*GGTA1* (α1, 3-galactosyltransferase)	ZFN	[77]
		LDLR (LDL receptor)	TALEN	[76]
	Bovine	*ACAN12, p65*	TALEN	[76]
	Human	*EMX1, PVALB*	CRISPR/Cas	[102]
	Rat	*IgM, Rab38*	ZFN	[70]
	Arabidopsis	*ADH1, TT4*	ZFN	[81]
	C. elegans	*ben-1, rex-1, sdc-2*	ZFN/TALEN	[78]
	Hamster	*DHFR*	ZFN	[37]
	Drosophila	*yellow*	ZFN	[72]
	Rice	*OsSWEET14*	TALEN	[84]
Gene addition	Human	*OCT4, PITX3*	ZFN/TALEN	[45,46]
	Human	*CCR5*	ZFN	[97]
	Human	*F9* (Coagulation Factor IX)	ZFN	[86]
	Mouse	*Rosa26*	ZFN	[57]
	Human	AAVS1	ZFN	[45,96,97]
			TALEN	[46]
			CRISPR/Cas	[103]
	Human	*VEGF-A*	ZFN	[17]
	Zebrafish	*th* (tyrosine hydroxylase), *fam46c, smad5*	TALEN	[80]
	Maize	*IPK1*	ZFN	[82]
Gene correction	Human	*IL2RG*	ZFN	[44,85]
		A1AT (α_1-antitrypsin)	ZFN	[89]
		HBB (β-globin)	ZFN	[87,88]
		SNCA (α-synuclein)	ZFN	[90]
	Tobacco	*SuRA, SurRB* (acetolactate synthase)	ZFN	[83]
	Drosophila	*yellow*	ZFN	[71]

mRNA-based methods for delivering ZFNs into cells are restricted to certain cell types and are associated with undesirable side effects, including insertional mutagenesis, toxicity, and low efficiency (Box 2). To address these limitations, we recently developed a simple alternative based on the direct delivery of purified ZFN proteins into cells. This approach does not carry the risk of insertional mutagenesis and leads to comparatively fewer off-target effects than ZFN gene-delivery systems that rely on expression from nucleic acids [65]. This type of delivery platform thus may represent an optimal strategy for studies that require precise genome engineering in cells.

Site-specific nucleases in model organisms

Site-specific nucleases have enabled the introduction of targeted modifications in several model organisms common to biological research, including zebrafish [66–68], rats and mice [69,70], *Drosophila* [71,72], *Caenorhabditis elegans* [73], and many other species for various applications, including the monarch butterfly [74], frogs [75], and livestock [76,77]. ZFNs and TALENs have also allowed investigators to compare gene function across related species, such as *C. elegans* and *Caenorhabditis briggsae* [78], shedding light on the similarities and differences between closely related organisms and making analyses between orthologous gene pairs possible. By microinjecting single-cell embryos with TALEN mRNA and single-stranded DNA oligonucleotides [79] or donor plasmid with extended (>800 bp) homology arms [80], TALENs have achieved targeted integration in zebrafish, enabling the generation of loxP engineered chromosomes and the possibility for conditional gene activation in this model organism. In addition to valuable animal models, both ZFNs and TALENs have been used to introduce targeted alterations in plants, including *Arabidopsis* [81] and several crop species [82,83], allowing the incorporation of valuable traits, such as disease [84] and herbicide resistance [82,83]. The diversity of organisms modified by these site-specific nucleases will undoubtedly continue to grow, expanding the repertoire of model systems for basic research and knowledge of the intricacies and opportunities of genome biology.

Therapeutic applications of site-specific nucleases

The use of site-specific nucleases for therapeutic purposes represents a paradigm shift in gene therapy. Unlike conventional methods, which either temporarily address disease symptoms or randomly integrate therapeutic factors in the genome, ZFNs and TALENs are capable of correcting the underlying cause of the disease, therefore permanently eliminating the symptoms with precise genome modifications. To date, ZFN-induced HDR has been used to directly correct the disease-causing mutations associated with X-linked severe combined immune deficiency (SCID) [85],

Box 2. Methods for delivering site-specific nucleases into cells

Although site-specific nucleases provide a means for introducing diverse custom alterations at specific genomic locations, this technology is still limited by methods for delivering these enzymes into relevant cell types. Typically, nuclease-encoded genes are delivered into cells by plasmid DNA, viral vectors, or *in vitro* transcribed mRNA. The delivery method can be tailored to some degree toward the application or cell type of interest; however, the deficiencies of contemporary viral and nonviral gene delivery systems restrict the possible applications of site-specific nucleases. In particular, transfection of plasmid DNA or mRNA by electroporation or cationic lipid-based reagents can be toxic and restricted to certain cell types. Viral vectors also present limitations, because they are complex, difficult-to-produce, potentially immunogenic, and involve additional regulatory hurdles. Despite these difficulties, clinical trials based on adenovirus-mediated ZFN gene delivery into T lymphocytes are ongoing [91], however, future endeavors would benefit greatly from improved delivery methods.

Integrase-deficient lentiviral vectors (IDLVs) are an attractive alternative for delivering ZFNs into transfection-resistant cell types [44]; however, this method does not appear to be compatible with highly repetitive TALEN sequences [107]. Despite the apparent ease with which TALENs can be engineered, these enzymes may prove more difficult to deliver into cells than ZFNs. AAV is a promising vector for ZFN delivery that has been used to enhance the efficiency of ZFN-mediated HDR [108,119] and drive ZFN-mediated gene correction *in vivo* [86]. Efficient packaging of AAV occurs only for expression cassettes less than 4.2 kb in length. Although this is sufficient to accommodate both ZFN monomers and an engineered donor construct, only a single TALEN monomer with a minimal promoter sequence can be inserted into this vector.

As an alternative to ZFN gene-delivery systems, our group recently reported that purified ZFN proteins are capable of crossing cell membranes and inducing endogenous gene disruption [65]. This approach has several advantages over gene-based delivery methods. First, this approach reduces off-target activity by limiting the time that cells are exposed to ZFNs and thus minimizing opportunities for off-target activity. Second, this method circumvents the cell-type dependency and toxicity of viral and nonviral gene delivery systems. Third, this approach overcomes several safety and regulatory hurdles for developing ZFN-based therapies by allowing the knockout of human genes without exposing cells to any genetic material. It remains unknown whether purified TALEN proteins can also introduced into cells in the same manner.

hemophilia B [86], sickle-cell disease [87,88], and α_1-antitrypsin deficiency [89]. Moreover, ZFNs have been used to genetically repair Parkinson's disease-associated mutations within the SNCA gene in patient-derived human iPS cells [90]. Targeted gene knockout via ZFN-induced NHEJ-mediated repair has also proven a potentially powerful strategy for combating HIV/AIDs. ZFNs have been used to confer HIV-1 resistance by disabling the HIV coreceptor C-C chemokine receptor type 5 (CCR5) in primary T cells [91] and hematopoietic stem/progenitor cells [92]. This approach is currently in clinical trials (NCT01252641, NCT00842634 and NCT01044654). More recently, ZFN-mediated targeted integration of anti-HIV restriction factors into the *CCR5* locus has led to the establishment of T cells that show near-complete protection from both R5- and X4-tropic strains of HIV [93]. Additionally, ZFNs have been used to improve the performance of T-cell-based immunotherapies by inactivating the expression of endogenous T cell receptor genes [94,95], thereby enabling the generation of tumor-specific T cells with improved efficacy profiles. Finally, site-specific nucleases afford the unique possibility of safely inserting therapeutic transgenes into specific 'safe harbor' locations in the human genome [96,97]. Although the overall utility of site-specific nucleases is currently limited in somatic cells, continued progress in stem cell research, including the production and manipulation of iPS cells, will ultimately open countless new directions for gene therapy, including treatments based on autologous stem cell transplantation.

Genome editing using programmable RNA-guided DNA endonucleases

Distinct from the site-specific nucleases described above, the CRISPR/Cas system has recently emerged as a potentially facile and efficient alternative to ZFNs and TALENs for inducing targeted genetic alterations. In bacteria, the CRISPR system provides acquired immunity against invading foreign DNA via RNA-guided DNA cleavage [98]. In the type II CRISPR/Cas system, short segments of foreign DNA, termed 'spacers' are integrated within the CRISPR genomic loci and transcribed and processed into short CRISPR RNA (crRNA). These crRNAs anneal to trans-activating crRNAs (tracrRNAs) and direct sequence-specific cleavage and silencing of pathogenic DNA by Cas proteins. Recent work has shown that target recognition by the Cas9 protein requires a 'seed' sequence within the crRNA and a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence upstream of the crRNA-binding region [99]. The CRISPR/Cas system can thereby be retargeted to cleave virtually any DNA sequence by redesigning the crRNA. Significantly, the CRISPR/Cas system has been shown to be directly portable to human cells by co-delivery of plasmids expressing the Cas9 endonuclease and the necessary crRNA components [100–103]. These programmable RNA-guided DNA endonucleases have demonstrated multiplexed gene disruption capabilities [102] and targeted integration in iPS cells [103]. Cas9 endonucleases have also been converted into nickases [102], enabling an additional level of control over the mechanism of DNA repair. In addition to human cells, CRISPR/Cas-mediated genome editing has been successfully demonstrated in zebrafish [104] and bacterial cells [105]; however, more exhaustive studies are required in order to thoroughly evaluate the utility of this system, including the potential for off-target effects. In particular, it remains unclear whether CRISPR/Cas system affords the requisite recognition selectivity necessary to ensure single-site specificity in complex genomes.

Concluding remarks and future directions

ZFNs, TALENs, and RNA-guided DNA endonucleases are transformative tools that have the potential to revolutionize biological research and affect personalized medicine. Indeed, these emerging technologies have dramatically expanded the ability to manipulate and study model organisms, and support the promise of correcting the genetic causes behind many diseases. However, in order to achieve the full potential of this technology, many important questions and challenges must be addressed (Box 3). Chief among these is the relative specificity of each nuclease platform. In the future, the use of high-throughput methods that enable comprehensive profiling of off-target cleavage sites [106] should provide insight into the stringency of

Box 3. Outstanding questions

- How effective are ZFNs and TALENs as therapeutic agents?
- What are the best methods for delivering site-specific nucleases into cells, and how can TALENs be delivered into cells by lentivirus?
- Can the Cas9 endonuclease be co-opted as a DNA-binding domain and be fused to enzymatic domains?
- How specific and safe are CRISPR/Cas9 systems, and how does the efficiency of Cas9-mediated genome editing compare to ZFN and TALEN-based approaches?
- What is the optimal RNA scaffold for application of CRISPR/Cas9 in mammalian cells?

target recognition inherent in each system. Questions also remain regarding the optimal methods for delivering these nucleases into cells and organisms. In particular, although adenoviral vectors can accommodate and deliver full-length TALEN genes into human cells, lentiviral plasmid vectors harboring TALEN sequences are prone to rearrangements after transduction [107]. Furthermore, the large size of TALENs may limit their delivery by size-restricted vectors such as recombinant adeno-associated virus (AAV), which has been shown to accommodate ZFN genes [108]. These findings suggest that the development of new TALEN delivery systems will be a critical area of future research. Although CRISPR/Cas systems show great promise and flexibility for genetic engineering, sequence requirements within the PAM sequence may constrain some applications. Directed evolution of the Cas9 protein should offer a path toward PAM independence, and may also provide a means to generate an even more efficient Cas9 endonuclease. Additional studies will also be required to evaluate the specificity and toxicity of RNA-guided DNA endonucleases *in vitro* and *in vivo*. Finally, the continued development of conditional methods that rely on customizable recombinases [109–111] and transcription factors [6,9,112–115] for affecting genomic structure and function will complement existing and future nuclease technologies. Together, these technologies promise to expand our ability to explore and alter any genome and constitute a new and promising paradigm to understand and treat disease.

Acknowledgments

The authors are supported by the National Institutes of Health (Pioneer Award DP1CA174426 (CB) and DP2OD008586 (CG) and National Science Foundation (CBET-1151035). T.G. was supported by National Institute of General Medicine Sciences fellowship (T32GM080209). We apologize to those investigators whose important contributions may have been omitted due to space constraints.

References

1 Capecchi, M.R. (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat. Rev. Genet.* 6, 507–512
2 McManus, M.T. and Sharp, P.A. (2002) Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* 3, 737–747
3 Urnov, F.D. *et al.* (2010) Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* 11, 636–646
4 Carroll, D. (2011) Genome engineering with zinc-finger nucleases. *Genetics* 188, 773–782
5 Wyman, C. and Kanaar, R. (2006) DNA double-strand break repair: all's well that ends well. *Annu. Rev. Genet.* 40, 363–383
6 Beerli, R.R. and Barbas, C.F., III (2002) Engineering polydactyl zinc-finger transcription factors. *Nat. Biotechnol.* 20, 135–141
7 Liu, Q. *et al.* (1997) Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5525–5530
8 Beerli, R.R. *et al.* (1998) Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14628–14633
9 Beerli, R.R. *et al.* (2000) Positive and negative regulation of endogenous genes by designed transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1495–1500
10 Kim, J.S. and Pabo, C.O. (1998) Getting a handhold on DNA: design of poly-zinc finger proteins with femtomolar dissociation constants. *Proc. Natl. Acad. Sci. U.S.A.* 95, 2812–2817
11 Segal, D.J. *et al.* (2006) Structure of Aart, a designed six-finger zinc finger peptide, bound to DNA. *J. Mol. Biol.* 363, 405–421
12 Neuteboom, L.W. *et al.* (2006) Effects of different zinc finger transcription factors on genomic targets. *Biochem. Biophys. Res. Commun.* 339, 263–270
13 Bhakta, M.S. *et al.* (2013) Highly active zinc-finger nucleases by extended modular assembly. *Genome Res.* 23, 530–538
14 Kim, S. *et al.* (2011) Preassembled zinc-finger arrays for rapid construction of ZFNs. *Nat. Methods* 8, 7
15 Gonzalez, B. *et al.* (2010) Modular system for the construction of zinc-finger libraries and proteins. *Nat. Protoc.* 5, 791–810
16 Segal, D.J. *et al.* (1999) Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5′-GNN-3′ DNA target sequences. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2758–2763
17 Maeder, M.L. *et al.* (2008) Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol. Cell* 31, 294–301
18 Sander, J.D. *et al.* (2011) Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat. Methods* 8, 67–69
19 Gupta, A. *et al.* (2012) An optimized two-finger archive for ZFN-mediated gene targeting. *Nat. Methods* 9, 588–590
20 Boch, J. *et al.* (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509–1512
21 Moscou, M.J. and Bogdanove, A.J. (2009) A simple cipher governs DNA recognition by TAL effectors. *Science* 326, 1501
22 Mak, A.N. *et al.* (2012) The crystal structure of TAL effector PthXo1 bound to its DNA target. *Science* 335, 716–719
23 Deng, D. *et al.* (2012) Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 335, 720–723
24 Christian, M. *et al.* (2010) Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186, 757–761
25 Mussolino, C. *et al.* (2011) A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res.* 39, 9283–9293
26 Miller, J.C. *et al.* (2011) A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* 29, 143–148
27 Zhang, F. *et al.* (2011) Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nat. Biotechnol.* 29, 149–153
28 Mercer, A.C. *et al.* (2012) Chimeric TALE recombinases with programmable DNA sequence specificity. *Nucleic Acids Res.* 40, 11163–11172
29 Cermak, T. *et al.* (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* 39, e82
30 Reyon, D. *et al.* (2012) FLASH assembly of TALENs for high-throughput genome editing. *Nat. Biotechnol.* 30, 460–465
31 Briggs, A.W. *et al.* (2012) Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. *Nucleic Acids Res.* 40, e117
32 Schmid-Burgk, J.L. *et al.* (2013) A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. *Nat. Biotechnol.* 31, 76–81

33 Kim, Y. *et al.* (2013) A library of TAL effector nucleases spanning the human genome. *Nat. Biotechnol.* 31, 251–258
34 Moehle, E.A. *et al.* (2007) Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3055–3060
35 Orlando, S.J. *et al.* (2010) Zinc-finger nuclease-driven targeted integration into mammalian genomes using donors with limited chromosomal homology. *Nucleic Acids Res.* 38, e152
36 Chen, F. *et al.* (2011) High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nat. Methods* 8, 753–755
37 Santiago, Y. *et al.* (2008) Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5809–5814
38 Lee, H.J. *et al.* (2010) Targeted chromosomal deletions in human cells using zinc finger nucleases. *Genome Res.* 20, 81–89
39 Sollu, C. *et al.* (2010) Autonomous zinc-finger nuclease pairs for targeted chromosomal deletion. *Nucleic Acids Res.* 38, 8269–8276
40 Lee, H.J. *et al.* (2012) Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. *Genome Res.* 22, 539–548
41 Brunet, E. *et al.* (2009) Chromosomal translocations induced at specified loci in human stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10620–10625
42 Cristea, S. *et al.* (2013) In vivo cleavage of transgene donors promotes nuclease-mediated targeted integration. *Biotechnol. Bioeng.* 110, 871–880
43 Maresca, M. *et al.* (2013) Obligate Ligation-Gated Recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining. *Genome Res.* 23, 539–546
44 Lombardo, A. *et al.* (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat. Biotechnol.* 25, 1298–1306
45 Hockemeyer, D. *et al.* (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat. Biotechnol.* 27, 851–857
46 Hockemeyer, D. *et al.* (2011) Genetic engineering of human pluripotent cells using TALE nucleases. *Nat. Biotechnol.* 29, 731–734
47 Ding, Q. *et al.* (2013) A TALEN genome-editing system for generating human stem cell-based disease models. *Cell Stem Cell* 12, 238–251
48 Zou, J. *et al.* (2009) Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell* 5, 97–110
49 Sanyal, A. *et al.* (2012) The long-range interaction landscape of gene promoters. *Nature* 489, 109–113
50 Gutschner, T. *et al.* (2011) Noncoding RNA gene silencing through genomic integration of RNA destabilizing elements using zinc finger nucleases. *Genome Res.* 21, 1944–1954
51 Dunham, I. *et al.* (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* 489, 57–74
52 Miller, J.C. *et al.* (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat. Biotechnol.* 25, 778–785
53 Szczepek, M. *et al.* (2007) Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat. Biotechnol.* 25, 786–793
54 Doyon, Y. *et al.* (2011) Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nat. Methods* 8, 74–79
55 Guo, J. *et al.* (2010) Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. *J. Mol. Biol.* 400, 96–107
56 Sood, R. *et al.* (2013) Efficient methods for targeted mutagenesis in zebrafish using zinc-finger nucleases: data from targeting of nine genes using CompoZr or CoDA ZFNs. *PLoS ONE* 8, e57239
57 Perez-Pinera, P. *et al.* (2012) Gene targeting to the ROSA26 locus directed by engineered zinc finger nucleases. *Nucleic Acids Res.* 40, 3741–3752
58 Doyon, Y. *et al.* (2010) Transient cold shock enhances zinc-finger nuclease-mediated gene disruption. *Nat. Methods* 7, 459–460
59 Certo, M.T. *et al.* (2012) Coupling endonucleases with DNA end-processing enzymes to drive gene disruption. *Nat. Methods* 9, 973–975
60 Kim, H. *et al.* (2011) Surrogate reporters for enrichment of cells with nuclease-induced mutations. *Nat. Methods* 8, 941–943
61 Kim, E. *et al.* (2012) Precision genome engineering with programmable DNA-nicking enzymes. *Genome Res.* 22, 1327–1333
62 Wang, J. *et al.* (2012) Targeted gene addition to a predetermined site in the human genome using a ZFN-based nicking enzyme. *Genome Res.* 22, 1316–1326
63 Ramirez, C.L. *et al.* (2012) Engineered zinc finger nickases induce homology-directed repair with reduced mutagenic effects. *Nucleic Acids Res.* 40, 5560–5568
64 McConnell Smith, A. *et al.* (2009) Generation of a nicking enzyme that stimulates site-specific gene conversion from the I-AniI LAGLIDADG homing endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* 106, 5099–5104
65 Gaj, T. *et al.* (2012) Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. *Nat. Methods* 9, 805–807
66 Doyon, Y. *et al.* (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.* 26, 702–708
67 Sander, J.D. *et al.* (2011) Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat. Biotechnol.* 29, 697–698
68 Meng, X. *et al.* (2008) Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat. Biotechnol.* 26, 695–701
69 Tesson, L. *et al.* (2011) Knockout rats generated by embryo microinjection of TALENs. *Nat. Biotechnol.* 29, 695–696
70 Geurts, A.M. *et al.* (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* 325, 433
71 Bibikova, M. *et al.* (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science* 300, 764
72 Bibikova, M. *et al.* (2002) Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics* 161, 1169–1175
73 Morton, J. *et al.* (2006) Induction and repair of zinc-finger nuclease-targeted double-strand breaks in *Caenorhabditis elegans* somatic cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16370–16375
74 Merlin, C. *et al.* (2013) Efficient targeted mutagenesis in the monarch butterfly using zinc-finger nucleases. *Genome Res.* 23, 159–168
75 Young, J.J. *et al.* (2011) Efficient targeted gene disruption in the soma and germ line of the frog *Xenopus tropicalis* using engineered zinc-finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7052–7057
76 Carlson, D.F. *et al.* (2012) Efficient TALEN-mediated gene knockout in livestock. *Proc. Natl. Acad. Sci. U.S.A.* 109, 17382–17387
77 Hauschild, J. *et al.* (2011) Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12013–12017
78 Wood, A.J. *et al.* (2011) Targeted genome editing across species using ZFNs and TALENs. *Science* 333, 307
79 Bedell, V.M. *et al.* (2012) In vivo genome editing using a high-efficiency TALEN system. *Nature* 491, 114–118
80 Zu, Y. *et al.* (2013) TALEN-mediated precise genome modification by homologous recombination in zebrafish. *Nat. Methods* 10, 329–331
81 Zhang, F. *et al.* (2010) High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12028–12033
82 Shukla, V.K. *et al.* (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature* 459, 437–441
83 Townsend, J.A. *et al.* (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature* 459, 442–445
84 Li, T. *et al.* (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* 30, 390–392
85 Urnov, F.D. *et al.* (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435, 646–651
86 Li, H. *et al.* (2011) In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* 475, 217–221
87 Zou, J. *et al.* (2011) Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. *Blood* 118, 4599–4608
88 Sebastiano, V. *et al.* (2011) In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. *Stem Cells* 29, 1717–1726
89 Yusa, K. *et al.* (2011) Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* 478, 391–394
90 Soldner, F. *et al.* (2011) Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell* 146, 318–331

91 Perez, E.E. *et al.* (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat. Biotechnol.* 26, 808–816
92 Holt, N. *et al.* (2010) Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. *Nat. Biotechnol.* 28, 839–847
93 Voit, R.A. *et al.* (2013) Generation of an HIV resistant T-cell line by targeted "stacking" of restriction factors. *Mol. Ther.* 21, 786–795
94 Torikai, H. *et al.* (2012) A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood* 119, 5697–5705
95 Provasi, E. *et al.* (2012) Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat. Med.* 18, 807–815
96 DeKelver, R.C. *et al.* (2010) Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res.* 20, 1133–1142
97 Lombardo, A. *et al.* (2011) Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nat. Methods* 8, 861–869
98 Wiedenheft, B. *et al.* (2012) RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 482, 331–338
99 Jinek, M. *et al.* (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821
100 Jinek, M. *et al.* (2013) RNA-programmed genome editing in human cells. *eLife* 2, e00471
101 Cho, S.W. *et al.* (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 230–232
102 Cong, L. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823
103 Mali, P. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823–826
104 Hwang, W.Y. *et al.* (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* 31, 227–229
105 Jiang, W. *et al.* (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.* 31, 233–239
106 Gabriel, R. *et al.* (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat. Biotechnol.* 29, 816–823
107 Holkers, M. *et al.* (2013) Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res.* 41, e63
108 Ellis, B.L. *et al.* (2013) Zinc-finger nuclease-mediated gene correction using single AAV vector transduction and enhancement by Food and Drug Administration-approved drugs. *Gene Ther.* 20, 35–42
109 Gaj, T. *et al.* (2013) A comprehensive approach to zinc-finger recombinase customization enables genomic targeting in human cells. *Nucleic Acids Res.* 41, 3937–3946
110 Gersbach, C.A. *et al.* (2011) Targeted plasmid integration into the human genome by an engineered zinc-finger recombinase. *Nucleic Acids Res.* 39, 7868–7878
111 Gaj, T. *et al.* (2011) Structure-guided reprogramming of serine recombinase DNA sequence specificity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 498–503
112 Beerli, R.R. *et al.* (2000) Chemically regulated zinc finger transcription factors. *J. Biol. Chem.* 275, 32617–32627
113 Polstein, L.R. and Gersbach, C.A. (2012) Light-inducible spatiotemporal control of gene activation by customizable zinc finger transcription factors. *J. Am. Chem. Soc.* 134, 16480–16483
114 Perez-Pinera, P. *et al.* (2013) Synergistic and tunable human gene activation by combinations of synthetic transcription factors. *Nat. Methods* 10, 239–242
115 Maeder, M.L. *et al.* (2013) Robust, synergistic regulation of human gene expression using TALE activators. *Nat. Methods* 10, 243–245
116 Yant, S.R. *et al.* (2007) Site-directed transposon integration in human cells. *Nucleic Acids Res.* 35, e50
117 Gersbach, C.A. *et al.* (2010) Directed evolution of recombinase specificity by split gene reassembly. *Nucleic Acids Res.* 38, 4198–4206
118 Owens, J.B. *et al.* (2012) Chimeric piggyBac transposases for genomic targeting in human cells. *Nucleic Acids Res.* 40, 6978–6991
119 Handel, E.M. *et al.* (2012) Versatile and efficient genome editing in human cells by combining zinc-finger nucleases with adeno-associated viral vectors. *Hum. Gene Ther.* 23, 321–329